

*AD*

GRANT NUMBER DAMD17-96-1-6073

TITLE: The Identification of Estrogen Responsive Genes in the Hypothalamic-Pituitary Axis

PRINCIPAL INVESTIGATOR: Caroline E. Connor  
Donald P. McDonnell, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center  
Durham, North Carolina 27710

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19971218 012

DTIC QUALITY INSPECTED

# REPORT DOCUMENTATION PAGE

*Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 96 - 30 Jun 97)		
4. TITLE AND SUBTITLE  The Identification of Estrogen Responsive Genes in the Hypothalamic-Pituitary Axis		5. FUNDING NUMBERS DAMD17-96-1-6073		
6. AUTHOR(S)  Caroline E. Connor Donald P. McDonnell, Ph.D.				
7. SPONSORING/MONITORING ORGANIZATION NAME(S) AND ADDRESS(ES)  Duke University Medical Center Durham, North Carolina 27710		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE		
13. ABSTRACT <i>(Maximum 200)</i>  <p>Estrogen mediates its biological responses through a specific interaction with the estrogen receptor (ER), an intracellular transcription factor which can bind specific DNA sequences and regulate gene expression. This process, however, is highly dependent on the cell and promoter context of the DNA-bound receptor, and our laboratory has demonstrated that the nature of the ligand also plays an important role. The identification of novel tissue-specific genes can therefore aid in the development of selective ligands for ER.</p> <p>Our laboratory is interested in defining estrogen-responsive genes in the hypothalamic-pituitary axis. Originally, this was to be accomplished using a yeast genetic selection system, developed in our laboratory, to identify promoter sequences which are transcriptionally activated in response to estrogen. Earlier use of this screen resulted in the cloning of numerous <i>Alu</i> sequences instead of classical estrogen-response elements (EREs). Unfortunately, our attempts to optimize this screen by altering receptor levels, using mutant receptors and treating with different ligands have not succeeded. Following the failure of an alternative method of gene identification, we are currently attempting to revisit the yeast genetic selection system using a novel approach.</p>				
14. SUBJECT TERMS Breast Cancer  estrogen receptor hypothalamic-pituitary axis		15. NUMBER OF PAGES 12 16. PRICE CODE		
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

All Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

ee For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Caroline E. Connor 7/28/97  
PI - Signature Date

PI - Signature

Date

## **TABLE OF CONTENTS**

I.	Front Cover	Page 1
II.	Report Documentation Page	Page 2
III.	Foreword	Page 3
IV.	Table of Contents	Page 4
V.	Introduction	Pages 5 - 6
VI.	Body	Pages 7 - 9
VII.	Conclusions	Page 10
VIII.	References	Pages 11 - 12

## INTRODUCTION

Breast cancer is the leading cause of cancer death among women and is expected to afflict one in nine of all American women (1). Among primary breast tumors, 60% have been shown to express the estrogen receptor (ER), which confers to the cell the ability to respond to estrogen (2). Although the role of ER in normal breast tissue remains unclear, it permits estrogen to exhibit a mitogenic effect on ER-positive breast cancer cells (3). Because of this action of estrogen, antiestrogens (synthetic compounds which block the actions of estrogen) have been clinically used to retard the growth of breast cancer tumors. Tamoxifen, the most widely used adjuvant therapy, albeit successful, results in undesirable side effects. Most effects are symptoms of estrogen withdrawal and include disturbances in vasomotor function and changes in the reproductive tract (4). One example is hot flashes, which can become so unbearable to the patient that a lack of compliance develops. These effects of estrogen on tissues are more clearly understood upon knowledge of the biology of ER.

The estrogen receptor is a nuclear protein and a member of the subfamily of steroid receptors which includes the progesterone, glucocorticoid, and androgen receptors (5, 6). There exist several functional regions that are highly conserved among all members of this superfamily at the nucleotide and amino acid level (5, 6). At the carboxyl terminus lies the hormone binding domain, a large region of approximately 250 amino acids that is hydrophobic in character. The DNA binding domain, which is the most highly conserved, consists of 66-68 amino acids including nine perfectly conserved cysteines. This domain forms two zinc fingers which confer sequence-specific DNA binding.

All steroid hormone receptors operate by a similar mechanism in which the latent receptor is bound in a multiprotein complex comprised of receptor, heat shock proteins, and additional cellular proteins (7). Upon ligand binding, these proteins are displaced and the receptor undergoes a conformational change and subsequent spontaneous dimerization (8). In this activated state the receptor can interact with specific DNA sequences (steroid response elements, or SREs) within target genes. The mechanism by which this event alters transcription of target genes remains to be elucidated. It is known, however, that antiestrogens are also capable of delivering the ER to DNA, but induce conformational changes in the receptor that do not allow for transcriptional activation (9).

The transactivation capacity of ER is mediated by two functionally distinct intramolecular regions: Activation Function 1 (AF1) in the amino terminus and AF2 in the carboxyl terminus (10, 11). Additionally, our lab has recently demonstrated that there is a third transactivation function, AF2a, which functions both in yeast and mammalian cells (12). These transactivation functions are differentially required in various cell contexts, so that ligands may preferentially activate one or more functions (13). This confers to the receptor the ability to differentially regulate distinct subsets of estrogen responsive genes. Because of the numerous biological functions that are controlled by estrogen, it is important to further define genes regulated by this hormone, with a view to developing antiestrogens with improved tolerance in patients. Therefore, we propose to identify estrogen responsive genes in the rat hypothalamic-pituitary axis which can function as surrogate markers of estrogen receptor action. This will permit a better understanding of the vasomotor actions of estrogen and the effects of anti-estrogen breast cancer therapies on this tissue.

Our laboratory has previously shown that ER can function as a hormone-dependent transcription factor in yeast (14, 15), and we have used this observation to develop a functional screen based on nutritional prototrophy to identify novel estrogen response elements (EREs) from a library of random oligonucleotides (16). The fact that these elements were shown to function indistinguishably in mammalian cells implied that this approach could be used to identify genomic DNA fragments which allowed ER action. In a yeast survival screen of genomic libraries from the MCF-7 breast cancer cell line and the

BRCA-1 breast cancer susceptibility gene locus (17), we identified a new subclass within the *Alu* family of DNA repeats which contained estrogen responsive elements (18). These *Alu* repeats apparently have diverged from known *Alu* sequences and acquired the ability to function as an ER-dependent enhancers in both yeast and mammalian cells.

However, we believe that the "classical" estrogen responsive enhancers, those which exhibit the classical ERE palindrome seen in the vitellogenin consensus sequence, comprise a larger, more important group of promoters than the *Alu* sequences. They are likely to have higher affinity for ER, and we are interested in optimizing our assay so that when we screen the rat hypothalamic-pituitary axis we do not simply clone lower affinity repetitive DNA. Following optimization and screening, we will analyze the pattern of expression of estrogen-induced mRNAs, as well as look at the ability of the various classes of antiestrogens to modulate this activity.

## BODY

### A. Optimization of Yeast Screen

#### Experimental Methods

The yeast screen was performed as previously done in our laboratory, with modifications made in hopes of optimizing the screen to select for classical EREs and not low affinity *Alu* sequences, under the assumption that the classical EREs would have more biological significance. One of the identified *Alu* clones, designated bcer-1 (18), was compared to the consensus vitellogenin sequence (19), designated wtERE. Both were contained within the *Bam*HI site of the pBM2389 parent vector. This low copy (ARS/CEN4) vector contains markers for TRP1 and Amp<sup>r</sup>, allowing for selection in yeast or bacteria. It also contains an enhancerless GAL1 promoter fused to the HIS3 coding sequence, so that the insertion of a functionally active enhancer (such as an ERE) into this vector and its subsequent transformation into yeast permits histidine prototrophy in a mutant *his3* background.

The plasmids were transformed into the *Saccharomyces cerevisiae* yeast strain, YPH500 (Mat  $\alpha$ , ura3-52, lys2-801, ade2-101, trp1 $\Delta$ 63, his3 $\Delta$ 200, leu2 $\Delta$ 1) (20) containing pRS-415-hER or YEPE22, a low copy and a high copy plasmid, respectively, expressing the wild-type human estrogen receptor. The yeast containing the low copy plasmid served as a positive control for growth, as these conditions were used previously. We chose to compare this growth to that of yeast containing a high copy receptor plasmid to see if altered receptor expression levels would affect growth. Transformants were plated on three sets of minimal media plates containing varying concentrations (1mM, 3mM, and 5mM) of aminotriazole, a competitive inhibitor of the HIS3 gene product, to select against sequences which resulted in weak activation of the HIS3 gene fusion. The first set of plates contained aminotriazole alone, to determine the amount of inhibitor to control for breakthrough growth. The second set of plates additionally contained histidine, and the third contained aminotriazole and 100nM 17 $\beta$ -estradiol. Five samples of liquid culture were plated of each sample, so that each plate contained both the wtERE and bcer-1 in YEPE22 and pRS-415-hER.

After five days at 30°, plates were examined for growth. Breakthrough growth was seen on plates containing 1mM or 3mM aminotriazole alone, but none was seen on those plates containing 5mM aminotriazole. Additionally, all colonies on all plates grew in the presence of histidine, and all colonies (except for one aberrant bcer-1 colony) grew in the presence of both aminotriazole and 17 $\beta$ -estradiol. As expected, there was slightly less growth (smaller colonies) seen with yeast containing the low copy versus the high copy ER. These results confirmed the validity of the experiment, as growth patterns were appropriate for enhancers previously determined to be estrogen responsive.

Another series of minimal media plates were made as previously indicated, using 5mM aminotriazole on each set, as this concentration appeared to be ideal for preventing breakthrough growth. However, the third set of plates was now made with six different estrogenic or antiestrogenic compounds as follows:

100nM 17 $\beta$ -estradiol  
10nM 4-OH tamoxifen  
100nM ICI 182,870  
100nM keoxifene  
100nM nafoxidine  
100nM GW7604

For this optimization assay, the YPH500 yeast contained one of each of the following ER constructs: YEPE22, pRS-415-hER, AF1 (in which the AF2 region of the receptor is deleted), AF2 (in which the AF1 region of the receptor is deleted) (11), and deletion 4 (a construct containing a mutation in another part of AF2). Yeast was transformed with the receptors and reporter constructs as mentioned above, so that each plate ultimately contained one liquid sample each of each receptor construct with each reporter construct (10 total per plate). This was done for each of the six hormones described, in hopes of finding a condition capable of splitting the screen to distinguish *Alu* sequences from classical EREs. After six days at 30°, however, no difference in growth between the wtERE and bcer-1 could be detected.

### Results and Discussion

The attempt to divide the yeast screen to preferentially select for high affinity, classical type EREs over low affinity *Alu* sequences was unsuccessful. We thought that perhaps estrogen-dependence varies as a result of receptor expression level, but this was not seen when yeast containing YEPE22 or pRS-415-hER were compared. An examination of enhancer sequences in the presence of mutant receptors did not reveal any different preferences for transactivation functions either. Since antiestrogens function less effectively in yeast, we hypothesized that they may only operate on the highest affinity enhancer elements. However, the conditions under which we screened using these various ligands did not result in any differential growth of our two enhancers.

### Recommendations

Unfortunately, our inability to optimize the yeast genetic selection system forced us to examine other options for identification of estrogen responsive genes. One method that seemed promising was that of whole genome polymerase chain reaction (WG-PCR) (21, 22). This method is used to screen genomic fragments for their ability to bind to a protein. The fragments are first "catch-linked" with an oligo, and once binding is accomplished, the bound fragments are removed, PCR amplified using the oligo as a primer, and rescreened for a determined number of cycles. The resulting fragments, which are expected to be high affinity binders, are subsequently analyzed. We decided that WG-PCR would be a viable option for us, as it would theoretically replace specific aims 1 and 2 of the proposal and allow immediate screening for EREs. Using ER as our protein, we could quickly screen the genome for high affinity binding sites, presumably getting rid of the weaker affinity *Alu* sequences during successive cycles. This methodology has been successful in the past in identifying estrogen responsive genes (23, 24).

## B. Whole Genome PCR

### Experimental Methods

We chose to use a nitrocellulose filter binding assay for use in our WG-PCR screening. Rat genomic DNA was partially digested with *Sau*3A and size fractionated using an agarose gel. Fragments from 500bp to 2000bp were ligated with specially designed catch linkers:



and purified on a Size Sep 400 column (Pharmacia) to remove excess linkers. Upon performing PCR using the smaller, unannealed oligo as a primer, a smear of DNA was seen, indicating that catch linked rat genomic DNA was present.

Before attempting the actual screen, we wanted to establish that the WG-PCR would work using the nitrocellulose filter binding assay. Two fragments were obtained from plasmids via PCR, one of which contained an ERE and one which did not. These were to serve as the positive and negative controls, respectively. The fragments were

radiolabeled with  $^{32}\text{P}$ -dCTP using Klenow enzyme. The GF/C Whatman filters were soaked in binding buffer, incubated (separately) with labeled positive and negative controls and purified ER, and then the filters were washed, dried, and counted. Conditions, including incubation times and temperatures, buffer conditions, and amounts of ER, were varied in hopes of achieving high retention of the ERE containing fragment on the filter and minimal retention of the non-ERE containing fragment. Unfortunately, the window was not as wide as anticipated. The introduction of herring sperm DNA into the reaction mixture to block non-specific binding was effective in reducing the amount of retention of non-ERE containing DNA, but also inhibited the retention of the positive control.

#### Results and Discussion

Despite intense manipulations of conditions and approaches, whole genome PCR did not seem to work. The window between our positive and negative controls was insufficient to validate that this approach would allow us to obtain EREs rather than non-specific DNA.

#### Recommendations

At this stage we have been unsuccessful in our attempt to identify estrogen responsive genes. Because the yeast screen is capable of determining estrogen responsive sequences, we wanted to devise a way to still use the screen but remove the odds of finding *Alu* sequences. Our approach currently entails the use of cDNA libraries from estrogen treated tissue, as it is rare that an *Alu* sequence is found in the coding sequence of a gene. The use of cDNA will allow us to search for intragenic estrogen responsive sequences such as those seen in PR and c-fos (25, 26). At this time we are preparing cDNA libraries for the screen.

## **CONCLUSIONS**

The identification of estrogen regulated genes is crucial for the understanding of the tissue-specific effects of the estrogen receptor. We are interested in the genes specifically regulated by ER in the hypothalamic-pituitary axis as a means to understanding the vasomotor disturbances which occur when women are treated with antiestrogens for breast cancer. Unfortunately, however, our efforts to date in identifying novel genes in this particular tissue have been frustrated by an inability to optimize our screening methods. We hope that our current technique of using cDNA in our yeast selection system will allow us to achieve our goals.

## REFERENCES

1. G. Bonadonna, *J. Clin. Oncol.* **7**, 1380-1397 (1989).
2. J. L. Wittliff, *Cancer* **53**, 630-643 (1984).
3. M. M. Gottardis, S. P. Robinson, V. C. Jordan, *J. Steroid Biochem.* **30**, 311-314 (1988).
4. R. R. Love, V. Koroltchouk, *Bull. World Health Organ.* **71**, 795-803 (1993).
5. S. Green, P. Chambon, *Trends Genet.* **4**, 309-314 (1988).
6. R. M. Evans, *Science* **240**, 889-895 (1988).
7. D. F. Smith, D. O. Toft, *Mol. Endocrinol.* **7**, 4-11 (1993).
8. D. P. McDonnell, D. L. Clemm, M. O. Imhof, *Semin. Cancer Biol.* **5**, 503.1-503.10 (1994).
9. D. P. McDonnell, D. L. Clemm, T. Hermann, M. E. Goldman, J. W. Pike, *Mol. Endocrinol.* **9**, 659-669 (1995).
10. L. Tora, et al., *Cell* **59**, 477-487 (1989).
11. M. T. Tzukerman, et al., *Mol. Endocrinol.* **8**, 21-30 (1994).
12. J. D. Norris, D. Fan, S. A. Kerner, D. P. McDonnell, *Mol. Endocrinol.* **11**, 747-754 (1997).
13. M. Berry, D. Metzger, P. Chambon, *EMBO J.* **9**, 2811-2818 (1990).
14. T. A. Pham, et al., *Proc. Natl. Acad. Sci. USA* **88**, 3125-3129 (1991).
15. T. A. Pham, Y.-P. Hwung, D. Santiso-Mere, D. P. McDonnell, B. W. O'Malley, *Mol. Endocrinol.* **6**, 1043-1050 (1992).
16. S. L. Dana, P. A. Hoener, D. A. Wheeler, C. B. Lawrence, D. P. McDonnell, *Mol. Endocrinol.* **8**, 1193-1207 (1994).
17. Y. Miki, et al., *Science* **266**, 66-71 (1994).
18. J. Norris, et al., *J. Biol. Chem.* **270**, 22777-22782 (1995).
19. L. Klein-Hitpass, M. Schorpp, U. Wagner, G. U. Ryffel, *Cell* **46**, 1053-1061 (1986).
20. R. S. Sikorski, P. Hieter, *Genetics* **122**, 19-27 (1989).
21. K. W. Kinzler, B. Vogelstein, *Nucleic Acids Res.* **17**, 3645-3653 (1989).
22. M. P. Costa-Giommi, M. Gaub, P. Chambon, P. Abarzua, *Nucleic Acids Res.* **20**, 3223-3232 (1992).
23. S. Inoue, S. Kondo, M. Hashimoto, T. Kondo, M. Muramatsu, *Nucleic Acids Res.* **19**, 4091-4096 (1991).
24. S. Inoue, et al., *Proc. Natl. Acad. Sci. USA* **90**, 11117-11121 (1993).
25. J. F. Savouret, et al., *EMBO J.* **10**, 1875-1883 (1991).

26. S. M. Hyder, G. M. Stancel, Z. Nawaz, D. P. McDonnell, D. S. Loose-Mitchell,  
*J. Biol. Chem.* **267**, 18047-18054 (1992).